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Galectin-3 regulates hepatic progenitor cell expansion during liver injury

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Short Title: Galectin-3 regulates hepatic progenitor cell expansion

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Abbreviations: ALT, Alanine transaminase; BMC, bone marrow cell; CDE, choline deficient-ethionine supplemented; DDC 3,5-diethoxycarbonyl-1,4-dihydrocollidine; EpCAM, Epithelial Cell Adhesion Molecule; Gal-3, galectin-3; gDNA, genomic DNA; HPC, Hepatic Progenitor Cell; NPC, non-parenchymal cells; pan-CK, pan-cytokeratin; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide

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Abstract

Objective: Following chronic liver injury or when hepatocyte proliferation is impaired, ductular reactions (DR) containing hepatic progenitor cells (HPC) appear in the periportal regions and can regenerate the liver parenchyma. HPCs exist in a niche composed of myofibroblasts, macrophages and laminin matrix. Galectin-3 (Gal-3) is a β-galactoside-binding lectin that binds to laminin and is expressed in injured liver in mice and humans.

Design: We examined the role of Gal-3 in HPC activation. HPC activation was studied following dietary induced hepatocellular (choline-deficient ethionine-supplemented diet) and billiary (3,5-diethoxycarbonyl-1,4-dihydrocollidine supplemented diet) injury in wild type and Gal-3(-/-) mice.

Results: HPC proliferation was significantly reduced in Gal-3(-/-) mice. Gal-3(-/-) mice failed to form a HPC niche, with reduced laminin formation. HPCs isolated from wild type mice secrete Gal-3 which enhanced adhesion and proliferation of HPCs on laminin in an undifferentiated form. These effects were attenuated in Gal3(-/-) HPCs and in wild type HPCs treated with the Gal-3 inhibitor lactose. Gal-3(-/-) HPCs *in vitro* showed increased hepatocyte function and prematurely upregulated both biliary and hepatocyte differentiation markers and regulated cell cycle genes leading to arrest in G0/G1.

Conclusions: We conclude that Gal-3 is required for the undifferentiated expansion of HPCs in their niche in injured liver.

Significance Statement

What is already known about this subject?
How might it impact on clinical practice in the foreseeable future

- Chronic liver disease accounts for 1.3% of all deaths worldwide.
- Following prolonged or severe liver injury ductular reactions (DRs) occur which contain hepatic progenitor cells (HPCs) which can differentiate into hepatocytes and biliary epithelium and can regenerate the liver parenchyma.
- Understanding the mechanisms that control DR expansion and HPC differentiation is crucial for the development of novel therapies to promote "healthy" liver regeneration.

What are the new findings?

- Our work has described the role of the beta-galactoside binding lectin galectin-3 (Gal-3) in the HPC niche.
- We show that Gal-3 facilitates DR expansion and promotes HPC interaction with laminin matrix and controls HPC differentiation.
- Mice deficient in Gal-3 display reduced HPC activation following biliary and hepatocellular injury.
- Gal-3 promotes the expansion of HPCs in an undifferentiated form.

How might it impact on clinical practice in the foreseeable future

- Gal-3 has an important role in the response to injury in the liver and inhibitors of Gal-3 function are being developed for liver fibrosis.
- A better understanding of its role in liver regeneration may lead to novel therapies for chronic liver disease.

Introduction

Liver cirrhosis accounts for 1.3% of all deaths worldwide and is a common precursor to the development of liver cancer which is the 3rd commonest cause of cancer death in men and 6th in women (WHO Global Burden of Disease Report 2004). In response to injury, the healthy liver restores its parenchymal mass through the division of mature liver cells- hepatocytes and bile duct cells. However following prolonged or severe liver injury hepatocyte mediated regeneration is impaired [1, 2] and ductular reactions (DRs) develop in the liver. DRs are thought to contain a population of hepatic progenitor cells (HPCs) which can differentiate under clonogenic conditions *in vitro* into hepatocytes and biliary epithelial cells [3]. DRs have been described in many forms of chronic human liver disease [4, 5, 6] and in severe acute liver disease [7]. Furthermore, a correlation exists between the extent of liver disease and the magnitude of the accompanying DRs [8]. Understanding the mechanisms controlling HPC activation is therefore important and may enable the development of therapeutic strategies to improve regeneration in chronic liver injury.

HPCs are surrounded by a "niche" composed of myofibroblasts, macrophages and the basement membrane which includes laminin [9]. We have shown that the cellular components of the HPC niche have an important effect upon the fate of HPCs [10] and both cell-cell and matrix-cell interactions within the niche are believed to influence HPC behaviour [9, 11, 12, 13]. Galectin 3 (Gal-3) is a 30kDa glycoprotein belonging to the β-galactoside-binding lectin family that is upregulated in injured liver [11, 14] and regulates myofibroblast activation and fibrosis [14]. Gal-3 has been reported to bind integrins and regulates β1 mediated adhesion to ECM [15, 16, 17] an interaction that can promote integrin signaling, adhesion, remodeling and fibronectin fibrillogenesis [18]. However no studies have examined the role of Gal-3 in DR/HPC activation. We have modelled two forms of liver injury; biliary injury and regeneration with the 3,5-diethoxycarbonyl-1,4-dihydrocollidine (DDC) diet, and hepatocellular injury with the choline deficient ethionine supplemented (CDE) diet [10]. Here we report that Gal-3 is highly expressed within the HPC niche, is actively secreted by HPCs and macrophages, is important for the interaction of HPCs with laminin and enables the expansion of HPC in an undifferentiated form.

Methods:

Animal Models

Animal work was carried out under procedural guidelines of the Home Office (UK). Mice with global and constitutive deletion of Gal-3 on a C57/Bl6J background [19] and strain matched C57/Bl6J WT mice from Harlan (UK) were used in this study. Eight-week old male mice were given 12 days of a choline deficient diet (MPbio, UK) supplemented with 0.15% DL-ethionine (Sigma-Aldrich, UK) in the drinking water [20]. Billiary activation was induced by administration of 0.1% 3,5-diethoxycarbonyl-1,4-dihydrocollidine (DDC) in the diet for 14 days. Animals undergoing partial hepatectomy were anesthetized with isofluorane. After a midline laparotomy, the left and middle lobes (~70%) of the liver were ligated at the base and removed. The abdominal wall and skin were sutured separately. BrdU was injected (50 mg/kg I.P.) 2 h prior to harvesting at 48h post hepatectomy.

Human Liver

Retrospectively collected specimens of human liver were obtained from the South East Scotland SAHSC BioResource

Immunohistochemistry

Formalin-fixed paraffin embedded sections were stained with picrosirius red and were processed for immunohistochemistry using the following antibodies: rabbit anti-PanCK (DAKO), rat anti F4/80 (eBioscience), rat anti-Gal-3 (Cedarlane), rabbit anti-BrDU (Abcam), rabbit anti-YM1 (Stem Cell Techologies) and the appropriate biotinylated secondary antibodies (DAKO) or alexa fluor conjugated antibodies (Invitrogen).

Cell counting and morphometric analysis of laminin deposition

For counting of DRs, 40 high power (x20) fields with liver parenchyma at all boundaries were counted. Ductular cells were recognized by the small (approximately 10μm) oval/cuboidal morphology with high nuclear to cytoplasmic ratio. Cells with hepatocyte like morphology (larger (> 20μm) with low nuclear to cytoplasmic ratio were not counted. For the quantification of proliferating ductular cells. Ki67 and PanCK double positive cells were counted from 10 high power (x20) fields. Laminin was scored from 40 high power (x20) fields. Livers from mice with partial hepatectomy were stained for BrdU and positive hepatocytes were counted from 20 (x20) fields.

HPCs purification and culture

Following DR/HPC induction with CDE diet, livers were harvested, minced, then incubated with L15 medium (Sigma) containing 50ug/ml DNase 1 (Roche), and 250μg/ml Collagenase type V (Sigma) at 37ºC in a shaking incubator for 45 min. Liver cells were then strained through a 40μm filter, centrifuged three times at 50 x g for 1 min to remove hepatocytes. The non-parenchymal cells were resuspended in HPC Complete Medium: 45% DMEM High Glucose (PAA), 45% Ham's F10 Medium (PAA,) 10% FBS (Hyclone), 1μg/ml Insulin, 50 μg/ml hydrocortisone (Sigma), 50 μg/ml gentamycin, 2.5 ml sodium pyruvate (PAA). HPCs were then purified by centrifugation through a discontinuous gradient of 20 and 50% Percoll™ (Sigma) in PBS at 1400 × g for 20 min. The lower layer was collected separately and washed twice with PBS. Leucocytes were depleted with anti-mouse CD45R/B220 magnetic particles (Beckton Dickenson). The negative fraction was resuspended in HPC Medium with 10% serum. To reduce fibroblast contamination, cells were plated for 8–12 h to remove adherent cells. Cells were replated and cultured in a humidified atmosphere with 5% CO_2 at 37 °C. LPC Medium with 5% serum and was replaced after 2 days. A more stringent protocol was also employed to isolate HPCs where HPCs were isolated by triple sorting for CD24, CD133 and EpCAM. Non-parenchymal cells were resuspended in PBS + 2% FCS and incubated with EpCAM-APC (eBiosciences); CD24-PeCy7, CD133-FITC, CD45-PE, CD31-PE and Ter119-PE (all from Biolegend). Hematopoeitic cells (CD45+), erythroid cells (Ter119), and endothelial cells (CD31+) were excluded by gating. 7-Aminoactinomycin D (7-AAD) was used to label dead cells for exclusion. Cells were analysed and sorted with FACS Aria II (Beckton Dickenson). Isolated cells formed colonies in collagen gels and single clones were isolated and expanded *in vitro*.

Primary HPCs proliferation and differentiation *in vitro*

WT and Gal-3(-/-) HPCs were cultured on laminin-coated or uncoated tissue culture plates, cells were fixed with methanol and stained for pan-CK. For HPC differentiation, cells were suspended in 2% matrigel in growth medium supplemented with 50 ng/ml epithelial growth factor (EGF, Sigma-Aldrich). Cells were fixed for staining and the total RNA were extracted after 1, 3, 5 days of induction.

Flow Cytometry

Primary non-parenchymal cells were incubated with mouse lineage cocktail (anti-Ter-119-PE, anti-CD45-PE, anti-CD31-PE), anti-CD34 PeCy7, anti-EpCam-APC (all from Biolegend) and viability dye eFluor 780 (EBiosciences) anti-mouse Gal-3-FITC (Mac-2, Cedarlane) or Gal-3-FITC plus anti-F480-APC and analysed using a LSR Fortessa II flow cytometer (Beckton Dickenson) and Flowjo software. Stellate cells were identified by autofluorescence [21].

Western Blotting

Cell extracts were resolved by SDS-PAGE and immunoblots probed overnight at 4°C with primary antibody, (rabbit anti-β-actin (Sigma Aldrich), rabbit anti-phospho-FAK, rabbit anti-Phospho-AKT, mouse anti-cyclin D1 (all from Cell Signaling), mouse anti-galectin-3 clone A3A12 (Alexis Biochemicals, UK) and appropriate horseradish peroxidase-lableled secondary antibodies (DAKO). Signal was detected using ECL reagent (GE Healthcare).

BMOL culture

BMOLs were kindly provided by Prof. George Yeoh and Dr Janina E E Tirnitz Parker. BMOLs were cultured in RPMI media with 5% FBS (PAA), 2mM L-glutamine (PAA), 10U/ml penicillin; 100ug/ml streptomycin (PAA), 30ng/ml IGF2 (Invitrogen), 20ng/ml EGF (Sigma) and 10ug/ml insulin (Sigma). Cells were cultured in a humidified atmosphere with 95% O_2 / 5% CO_2 at 37°C.

BMDM isolation and co-culture

BMDMs were isolated as previously described (Mackinnon et al., 2008) from WT or Gal-3(-/-) mice and cultured in DMEM/F12 media containing 20% L929 conditioned media and 10% FBS. For co-culture experiments BMOL cells and BMDMs were washed and incubated overnight prior to seeding together at a 1:1 ratio in BMOL growth media. Cells were cultured for 72h prior to MTT assay (see below).

siRNA Transfection

SiRNA-mediated knockdown of mouse Gal-3 expression was carried out using duplexes against the target sequence CACAATCATGGGCACAGTGAA. Transfection with Hiperfect (Qiagen) was performed according to the manufacturer's protocol.

Immunofluorescence

HPCs were immunostained using routine methods, primary antibodies rabbit anti-pan-cytokeratin (DAKO), rabbit anti-sox-9 (Abcam), rat anti-Ki67 (Leica), mouse anti-alpha-SMA (Sigma) and rat anti-Gal-3 (Cedarlane) were used in 1% BSA in PBS for 1hr. Secondary antibodies were donkey anti-rabbit 488, goat anti-rabbit 568 or donkey anti-rat 488 (1:250 in 1% BSA in PBS).

Quantitative real-time RT-PCR analysis.

RNA was prepared using RNeasy (Qiagen), as per the manufacturer's protocol. 100ng RNA was used for cDNA synthesis using Superscript II First Strand Synthesis System (Invitrogen), with random hexamer primers. Quantitative real-time PCR was performed for cDNA samples using SYBR Green master mix (Qiagen) and Quantitect primers (Qiagen) as follows; Gamma-glutamyl transpeptidase (γGT, Cat. No. QT00104209), aquaporin-1 (Cat. No. QT00109242), hepatocyte nuclear factor 4 (HNF-4, Cat. No. QT00144739), albumin (Cat. No. QT00115570), cytokeratin-19 (Cat. No. QT00156667).

PCR array

cDNA from control duplex and galectin-3 siRNA transfected BMOL cells was applied to a mouse cell cycle RT2 Profiler PCR Array (SABiosciences PAMM-020Z) as per manufacturer's instructions. Data analysis was carried out using an integrated web-based software package (SA Biosciences) based on the $2^{-\Delta\Delta CT}$ method with normalization of the raw data to GapDH and is expressed as fold regulation compared to cells transfected with scrambled duplex.

MTT Cell Proliferation Assay

Cells were plated (7.5×10^3 cells / well) in replicates of 6 in 96-well culture plates and treated with 50 mM lactose or 50 mM sucrose for 2 or 4 days. To measure the cell proliferation, 20µl of 5 mg/ml MTT (Sigma) was added into each well and assayed using standard methodology.

Cell Adhesion Assay

Cell adhesion was assayed using BMOL cells on plastic culture plates coated with either laminin or 10% poly-L-lysine for positive control at 37˚C for 2 hour. After incubation, the cells were fixed and stained with Diff-Quick. The plate was washed with water then 50µl DMSO was added into wells. The plate was then read in a plate reader at 660 nm wavelength.

ELISA Assay

Mouse serum albumin and Gal-3 were measured by ELISA (albumin; Alpha Diagnostics, Gal-3 Duoset kit, R&D Systems) according to the manufacturer's instructions.

Cell Cycle analysis

Cell cycle stage was measured by DNA content analysis by flow cytometry following labeling of methanol fixed cells with propidium iodide. Doublets were excluded by analysis of FL2-area against FL2-width using a FacsCaliber (Beckton Dickenson) and Flowjo software.

Measurement of P450 enzyme activity

Functional analysis of cytochrome p450 activity (CYP1A2) was performed using p450-Glo systems according to the manufacturer's protocol (Promega).

Results

Hepatic Gal-3 expression is upregulated and associated with the CDE-diet induced DRs.

Bile ducts from WT mice were identified by pan-cytokeratin (pan-CK, Figure 1A). Gal-3 was localised mainly to macrophages and some bile duct epithelia in control diet mice. WT mice fed a CDE diet showed typical peri-ductular reaction with pan-CK positive ductular cells migrating into the parenchyma away from periportal area. Mice fed the CDE diet for 12 days showed significantly higher liver Gal-3 mRNA levels than control-diet fed mice (Figure 1B) and numerous Gal-3 positive cells were observed in the peri-portal region of CDE-fed mouse livers. Dual immunofluorescence staining showed a spatial association between the DRs and Gal-3 (Figure 1C). Gal-3 was strongly expressed in macrophages within the niche and also in some pan-CK positive progenitor cells and hepatocytes surrounding the niche (Figure 1C, supplemental Figure S1A). A strong association between DRs and Gal-3 was also observed in human cirrhotic liver of diverse aetiologies including diseases affecting the biliary system (primary sclerosing cholangitis, PSC and primary biliary cirrhosis, PBC) and hepatocellular injury (Hepatitis C virus infection, HCV) where Gal-3 was expressed on and around pan-CK positive DRs (Figure 1D).

Gal-3 is critical for ductular expansion and proliferation *in vivo* and associated niche formation

WT and Gal-3(-/-) mice were given CDE diet for 12 days. Gal-3(-/-) livers showed only few pan-CK positive cells and these were almost exclusively bile ductular cells located in the periportal tracks (Figure 2A). This was also confirmed when livers were stained with sox-9 (Figure 2A lower panels). The number of ductular cells was significantly decreased in Gal-3(-/-) mice (Figure 2B $p<0.05$). Importantly, the absence of a ductular response in Gal-3(-/-) livers was not associated with protection from the hepatotoxic effects of the CDE diet on the mice. In contrast, serum transaminase activity was higher in Gal-3(-/-) mice compared to WT (Figure 2C). Further, other parameters of morbidity including weight loss, matting of the fur and withdrawal from socialisation were routinely monitored, and were not distinguishably different between strains. To address whether the reduced number of DRs in Gal-3(-/-) mice was associated with reduced proliferation of ductular cells, we conducted dual-staining for Ki67 and pan-CK to determine the *in situ* proliferative index of DRs in CDE treated livers (Figure 2D). Pan-CK/Ki67 dual positive cells were observed in WT but not Gal-3(-/-) CDE treated livers (Figure 2D) and the proliferative index was 7 fold higher in WT compared to Gal-3(-/-) mice confirming that the proliferation of

these cells was affected by the absence of Gal-3 (Figure 2E $p<0.05$). The number of proliferating hepatocytes however was low and was not markedly different between genotypes (Supplemental Figure S2A). _We immunostained sections for typical HPC niche components and showed that unlike WT mice, Gal-3(-/-) mice failed to form a typical laminin sheath that surrounds HPCs radiating from the periportal area (Figure 2F). The amount of laminin in CDE-fed Gal-3(-/-) mouse livers was significantly less than that in CDE-fed WT mouse livers (Figure 2G, $P<0.01$). The number of infiltrating macrophages was reduced in Gal-3(-/-) mice compared to WT (Figure 2F,G). This was due to a reduced number of M2 activated macrophages as cells positive for the M2 marker YM-1 were reduced in the Gal-3(-/-) mice compared to WT (Supplemental Figure S2B). Following biliary injury with DDC diet YM-1 positive cells were seen around the areas of the ductular reaction and on Kupffer cells within the parenchyma. Ductular YM-1 positive cells were not observed in the Gal-3(-/-) livers. Recruitment of neutrophils and CD3 positive T cells was not significantly altered following CDE diet and there was no reduction in Gal-3(-/-) mice. (Supplemental Figure S3).

In WT mice, biliary injury induced by DDC diet resulted in ductular proliferation with periductal fibrosis (Figure 3). Numerous pan-CK-positive and sox-9 positive ductular cells were seen around portal tracts (Figure 3A). In contrast there were fewer ducts/portal tract and reduced fibrosis in Gal-3(-/-) mice compared to WT (Figure 3B,C). Dual immunofluorescence staining revealed that Gal-3 was expressed on cells surrounding the proliferating ductular cells and on some ductular cells themselves (Figure 3D). However significant co-staining for Gal-3 was observed on alpha-smooth muscle actin positive stellate cells and macrophages following DDC diet (Supplemental Figure S1A). Moreover flow cytometric analysis showed that Gal-3 was expressed both on F480-positive macrophages and stellate cells from non-parenchymal cells isolated from DDC treated WT livers (Supplemental Figure S1B).

Our results suggest a role for Gal-3 in progenitor mediated liver regeneration, therefore we sought to examine the role of Gal-3 in hepatocyte driven regeneration following partial hepatectomy. Liver weights and the number of proliferating BrdU positive hepatocytes was not significantly different between WT and Gal-3(-/-) mice (Supplemental Figure S4).

Gal-3 promotes HPC adhesion and proliferation on laminin

We isolated HPCs from CDE-fed mouse livers. FACS analysis showed that lineage negative, CD24 and EpCAM positive HPCs expressed Gal-3 (Figure 4A). On day 4 post isolation HPCs

were > 95% positive for sox-9 and pan-CK (Figure 5B) and co-expressed Gal-3 diffusely within the cytoplasm (Figure 4B). Using a second method we isolated highly sorted HPCs (CD45-/CD31-/Ter119-/CD24+/EpCAM+/CD133+) from the non-parenchymal fraction of livers of mice that had received the CDE diet (supplemental Figure S5A). We have previously shown that these cells are clonogenic and have bipotential differentiation capacity yielding cells of a hepatocyte and biliary morphology *in vitro* at clonal density [22]. We confirmed that this highly sorted population could be differentiated down a biliary or hepatocellular lineage (Supplemental Figure S6B). Yields of CD24+CD133+EpCAM+ HPCs were 2.8% of the lineage negative fraction in WT livers and 1.7% in Gal-3(-/-) livers. Transfection of these cells with siRNA to Gal-3 increased the spontaneous differentiation of these cells down a hepatocellular or biliary fate (supplemental Figure S5C).

WT HPCs when plated on laminin rapidly adopted an epithelial morphology (Figure 4C) and up-regulated expression and secretion of Gal-3 (Figure 4D). BMOL cells also adhered readily to laminin (Figure 4E). This adhesion was significantly inhibited by lactose, an extracellular Gal-3 inhibitor, whereas no effect was seen with an equimolar concentration of sucrose (Figure 4E). Primary HPCs from WT mice plated on laminin showed significantly increased proliferation compared to those maintained on plastic (Figure 4F). Similar results were obtained with the BMOL cell line (Figure 4G).

Absence/inhibition of Gal-3 inhibits proliferation of HPC on laminin

To determine the effects of extracellular Gal-3 upon HPC proliferation we examined the effect of lactose and sucrose on primary HPC proliferation by MTT assay. Lactose inhibited growth of WT HPCs on laminin compared to sucrose control or untreated cells (Figure 5A). Gal-3(-/-) HPCs grew at a significantly slower rate than WT cells (Figure 5B). The effect of Gal-3 on cell growth suggested that Gal-3 may modulate cell cycle progression. To address this we used BMOL cells transfected with siRNA to Gal-3. siRNA transfection resulted in >90% knockdown of Gal-3 expression measured by qPCR, flow cytometry, ELISA and western blot (Figure 5C). Transfected cells were analysed for cell cycle progression by DNA content analysis by propidium iodide staining (Figure 5D). At 72h post transfection inhibition of Gal-3 expression resulted in a significant reduction in the percentage of cells in G2 (from 25 to 13%) indicating a block in cell cycle progression at the G_0/G_1 phase of the cell cycle (Figure 5D). To further investigate effects on the cell cycle, a mouse cell cycle PCR array was performed on RNA isolated from transfected BMOLs plated on laminin. Inhibition of Gal-3 expression in BMOL

cells resulted in decreased expression of >1.5 fold of several key cell cycle regulatory genes including cyclinD1, CDK1, CDK6, cdc6, Chek1, and Rbl1 and decreased expression of mitotic assembly genes NEK2 and RAD21 coupled with an increased expression of checkpoint genes Ddit3 and CKS1b and the cell cycle inhibitory gene P16 (Figure 5E). To explore this further we examined changes in the expression of cyclinD1 in primary HPCs and showed a reduction in expression in Gal-3(-/-) HPCs (Figure 5F). We also show that Gal-3 deletion results in reduced activation of the downstream integrin signaling intermediates focal adhesion kinase (FAK) and Akt (Figure 5F). These results are consistent with Gal-3 mediating adhesion of HPCs in the niche which facilitates integrin engagement and signaling leading to cell cycle progression.

Gal-3 facilitates the expansion of HPCs in an undifferentiated phenotype

We then sought to examine whether inhibition of Gal-3 expression induced differentiation of HPCs. To induce bi-lineage differentiation of HPCs, a combination of matrigel and EGF was employed [23]. Differentiation of HPCs plated on plastic rapidly invoked changes in their gene expression consistent with maturation into terminally differentiated epithelium. After 5 days in culture this resulted in upregulation of both hepatic and biliary markers in BMOLs plated on plastic, whereas culture on laminin (with matrigel and EGF) suppressed this effect (Supplemental Figure S6). This suggests that laminin enables the maintenance of HPCs in an undifferentiated form *in vitro*. In contrast, both primary HPC isolates (Figure 6A-E) and BMOLs subjected to siRNA knockdown (Supplemental Figure S6F-K) differentiated readily on laminin. Interestingly, there was no apparent lineage-bias induced by the absence of Gal-3; both mature biliary and hepatocytic genes were upregulated in knockout/knock down cultures compared to control. Furthermore, hepatocyte functional assays were performed by measuring albumin secretion and CYP1A2 activity. These data demonstrate that there is a significantly higher albumin secretion and CYP1A2 activity in Gal-3 siRNA transfected BMOLs than in control transfected cells. (Figure 6F,G) These data suggest that Gal-3, together with laminin, is important for maintaining the undifferentiated state of HPCs in the hepatic niche.

Macrophages contribute to the Gal-3 composition of the HPC niche

Macrophages are a major Gal-3 expressing cell component of the HPC niche in the regenerating liver. Culture supernatants from bone marrow derived macrophages (BMDMs) plated for 24h contained five times as much Gal-3 as that produced by equal numbers of HPCs (Figure 7A). BMDMs from WT or Gal-3(-/-) mice were co-cultured with BMOL cells and proliferation measured by MTT assay. Co-culturing control BMOLs for 3 days with either WT or

Gal-3(-/-) BMDMs had no effect on BMOL proliferation. However when Gal-3 expression was inhibited in BMOLs there was reduced BMOL proliferation which could be at least partially rescued by co-culturing with WT macrophages (Figure 7B).

Discussion

Gal-3 is a beta galactoside binding lectin most often associated with inflammation and fibrotic injury in various tissues, including liver [14, 24, 25] and is elevated in chronic liver injury in mice and humans [11, 14] and has been associated with worsening fibrosis, with Gal-3(-/-) mice showing reduced scarring. In acute injury Gal-3(-/-) mice show reduced hepatitis following concanavilin-A [26] and acetaminophen [27, 28] induced injury. Therefore in most of these studies the absence of Gal-3 has been associated with beneficial outcome and scar free repair. However none of these models result in a significant DR and impaired hepatocyte proliferation similar to that seen in many forms of chronic human liver disease.

Here we show that Gal-3 is elevated in the CDE model of liver injury with DRs/HPC activation and hepatocyte regeneration. We found numerous Gal-3 expressing cells localized to and around the DRs spreading from the periportal area of the liver. In mouse liver, we observed Gal-3 positive cells within the niche but was primarily associated with macrophages rather than the HPCs themselves. Gal-3 also known as Mac-2 has been used as a macrophage marker in several tissues [29]. Expression of Gal-3 in niche macrophages suggests that Gal-3 may be involved in cross-talk between HPCs and their supporting environment, which is believed to be crucial in mediating the homeostatic balance between proliferation and differentiation of HPCs during liver regeneration [9, 10, 22, 30]. We also observed a population of Gal-3 positive hepatocytes in CDE-treated mice. We could speculate that's these cells are newly formed hepatocytes arising from differentiated progenitors but this would require further confirmation. Gal-3 positive hepatocytes were also observed in human liver (although clearly less strongly positive than the ducts), This may reflect differences in the chronicity of the diseases in humans and mice.

We found that Gal-3(-/-) mice demonstrated reduced macrophage infiltration and lacked a DR response to the CDE diet, despite showing increased liver injury. The reduced number of macrophages in Gal-3-/- livers may result in reduced clearance of ALT leading to elevated levels in the serum [31]. The number and the proportion of proliferating ductular cells was significantly reduced in Gal-3(-/-) mice indicating a direct effect of Gal-3 upon cell proliferation.

Similarly, DRs were reduced in Gal-3(-/-) mice following biliary injury with the DDC diet. In the DDC diet hepatic stellate cell-derived Gal-3 may contribute to the progenitor expansion. This confirms our previous work [10] which demonstrates that myofibroblasts associate with proliferating ducts during billiary injury but not hepatocellular injury (CDE). In contrast we show

that deficiency of Gal-3 has no effect on hepatocyte mediated regeneration following partial hepatectomy.

Laminin forms a dynamic sheath flanking proliferating DR/HPCs, and unlike other matrices permits HPC proliferation in an undifferentiated form [9, 32] and discussed in [33]). We show that laminin facilitates proliferation and suppresses differentiation of HPCs and that these effects are promoted by Gal-3. Absence or knockdown of Gal-3 promoted differentiation into a more mature phenotype with expression of both biliary and hepatocyte markers. We show that HPCs in culture secrete Gal-3 and that lactose inhibits Gal-3 mediated adhesion and proliferation on laminin. The mechanism whereby Gal-3 mediates its effect on HPC proliferation is not completely understood but it is known to directly bind and activate cyclin D1 in the nucleus [34, 35, 36, 37]. We showed that there was reduced expression of cyclin D1 by western blot in Gal-3(-/-) HPCs and found that growth of Gal-3 knockout/down HPCs on laminin was slower *in vitro* and there was an accumulation of cells in G1/0 and a reduction in the % of cells in G2/M consistent with cell cycle arrest in G1/0. There was a reduction in expression of cell cycle regulatory molecules, cyclins and CDKs and reduced expression of proteins involved in mitosis and chromosome assembly (RAD21 and NEK2) and an increased expression of the cell cycle inhibitor P16 and checkpoint protein Ddit3.

Most adherent cells require integrins to progress through the cell cycle either by controlling growth factor signaling or by activating enzymes that are necessary for the G1 phase of the cell cycle [38]. Gal-3 has been shown to directly bind □1-integrin and modulates adhesion to ECM components [39, 40, 41] and has been shown to stabilise FAK in focal adhesions and increase FAK and Akt phosphorylation [18, 42]. We show that integrin-mediated signaling was inhibited in Gal-3(-/-) HPCs compared to WT cells when plated on laminin. This could be due to binding of Gal-3 to its putative membrane receptor CD98 [43, 44] which can modulate integrin function by a direct interaction with □1-integrin tails [44, 45]. There are also several studies that implicate an intracellular signaling role for galectin-3 [36, 46, 47] and Gal-3 has been shown to directly modulate cyclins [36]. Our work showing that the effect of Gal-3 in the context of HPC proliferation and adhesion and the role of FAK all of which are inhibited by lactose suggest primarily an extracellular effect.

We show that co-culture with Gal-3 expressing macrophages could restore proliferation of Gal-3 depleted HPCs *in vitro*, suggesting that recruited cells contribute to the Gal-3 expression within

the niche. We propose that Gal-3 (secreted by HPCs and niche macrophages) stimulates DR/HPC expansion by supporting adhesion to the laminin sheath and activating integrin signaling leading to cell cycle progression (Supplemental Figure 8).

How would a proliferative effect on HPCs impact on fibrosis? On one hand matrix deposition (principally laminin) is necessary for HPC proliferation, whilst the ductular reaction promotes excessive matrix deposition leading to fibrosis. We have shown that these processes can be manipulated in opposing directions by macrophages which could be explained by differing matrix responses at each pole of the ductular reaction (biliary vs hepatocytic) [10, 22, 48]. The differential regulation of these processes would impact on the eventual outcome. Our results coupled with the well described profibrotic role of Gal-3 would support a hypothesis that suggests that Gal-3 within the niche stimulates HPC expansion. HPCs emerging from the niche would be exposed to a low Gal-3 environment that would favour differentiation but upon persistent injury leads to matrix deposition and fibrosis.

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The authors declare no conflict of interest.

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Figure Legends

Figure 1 – Gal-3 expression is up-regulated during DR expansion *in vivo*. (A). Representative sections of liver from control and CDE fed mice immunostained for ductular cells (pan-CK) and Gal-3 (scale bar = 50μm). (B) Whole liver gene expression of Gal-3 was significantly elevated in 12-day CDE diet fed mice versus controls (n=6, P<0.05). (C) Dual staining for pan-CK (red) and Gal-3 (green) following 12 days CDE diet showing Gal-3 positive cells in periportal zones of the ductular reaction. Arrows designate dual positive cells (scale bar = 10 μm). (D) Representative immunostaining for Gal-3 and pan-CK in sections from normal human liver and liver section from patients with primary biliary cirrhosis (PBC), hepatitis virus C (HCV) and primary sclerosing cholangitis (PSC). Arrows indicate Gal-3 expression within DRs (scale bar = 50 μm).

Figure 2 – Gal-3 knockout mice have reduced DR proliferation in response to a CDE diet. (A) Pan-CK and sox-9 staining in livers from WT and Gal-3(-/-) mice fed CDE diet for 12 days (scale bar = 50 • m). (B) Quantification of pan-CK staining showed consistently fewer HPCs in the livers of Gal-3(-/-) mice fed CDE diet than those in the equivalent WT (n=6, **P<0.01). (C) Serum alanine transaminase (ALT) activity was higher in Gal-3(-/-) mice compared to WT at the end of the experiment. (D) Ki67 staining yielded significantly fewer positive nuclei, suggestive of reduced HPC proliferation in Gal-3(-/-) mice (scale bar = 25 • m). (E) This was confirmed by dual-staining and counts of pan-CK (green) and Ki67 (red). Arrows indicate double-positive cells. Both overall ductular cell number and the proliferative index of ductular cells were significantly lower in Gal-3(-/-) compared to WT (Student's T-test, n=6; ** P<0.01). (F) Immunostaining for laminin was performed on serial sections of liver from wild type (WT) and Gal-3(-/-) mice fed CDE diet for 12 days. Representative immunostaining of the same area in each liver is illustrated (scale bar = 25 μm). Gal-3(-/-) mice failed to form all elements of the HPC nice following CDE diet induction. (G) Laminin and macrophage scoring analysis showed a significant lower expression of laminin and reduced macrophage accumulation in CDE-fed Gal-3(-/-) mice liver compared to WT controls (** P<0.01, n=6).

Figure 3 – Gal-3(-/-) mice show reduced DR in response to DDC diet. Wild type (WT) and Gal-3(-/-) mice received DDC supplemented diet for 14 days. (A) Sections of WT and Gal-3(-/-) livers stained for pan-CK, sox-9 and sirius red (scale bar = 50μm). Digital image analysis quantification of pan-CK (B) and collagen (sirius red) staining (C) showing fewer pan-CK positive ductular cells were around portal tracts in Gal-3(-/-) mice compared to WT with reduced fibrosis (* P<0.05, n=5). (D) Co-immunofluorescence staining for Gal-3 (green) and pan-CK (red) in WT (left) and Gal-3(-/-) mice (right). Arrows show dual positive cells.

Figure 4 – Gal-3 promotes HPC adhesion to laminin. (A) Cells from collagenase digested WT CDE-fed mouse livers were analysed by flow cytometry. Lineage$^-$EpCAM$^+$CD24$^+$ cells (histogram) were positive for Gal-3. (B) Leucocyte and endothelial depleted cells were cultured for 4 days and immunostained for pan-CK (red) and Gal-3 (green) left panel, and sox-9 (red) and Gal-3 (green) right panel (scale bar = 25μm). Nuclei were stained with DAPI. (C) Phase contrast images of HPCs adhered to plastic or laminin coated plates showing spreading and epithelial morphology on laminin. Original magnification: x 200. (D) Gal-3 expression measured by ELISA was increased in HPCs plated on laminin for 24h. (E) Pre-treatment of BMOL cells with 50 mM lactose (lac) but not 50 mM sucrose (suc) significantly reduced adhesion to laminin

(LM, *** P<0.001, n=5). Primary (F) or BMOL (G) HPCs were cultured on laminin or plastic and their proliferation assayed by MTT assay (**P<0.01, n=5).

Figure 5 – Absence of Gal-3 inhibits proliferation of HPCs on laminin. (A) MTT assay showing inhibition of primary WT HPC growth on laminin with 50 mM lactose but not sucrose (n=4, ** P<0.01). (B) Cell counts of primary HPCs from Gal-3(-/-) and WT mice plated for 4 days on laminin (n=4, *P<0.05, compared to WT). (C) BMOLcells were transfected with siRNA to Gal-3 or control duplex and after 72h Gal-3 knockdown was assessed by ELISA (n=4), qPCR (n=4), flow cytometry and western blot (representative of 4 independent experiments). (D) Control and Gal-3 siRNA treated HPCs were fixed and DNA content assessed by propidium iodide staining and flow cytometry after gating out doublets. Gal-3 siRNA treated cells showed decreased % of cells in G2/M and increased % of cells in G1 (representative trace from 4 experiments). (E) Transcripts from control transfected and Gal-3 siRNA transfected HPCs 72h post transfection was analysed using a mouse cell cycle RT-profiler PCR Array. Results are expressed as fold regulation compared to control. The results are the mean $\pm$ SEM of 4 arrays. (F) Primary HPCs from WT and Gal-3(-/-) mice were cultured on laminin for 4 days and lysates probed for cyclin D1 and phosphorylated FAK and Akt, Gal-3 and β-actin. Absence of Gal-3 conferred reduced cyclin-D protein levels and reduced phosphorylation of FAK and Akt.

Figure 6 – Gal-3 is required for the maintenance of an undifferentiated phenotype on laminin. Primary HPCs were isolated and cultured on laminin under differentiating conditions (matrigel and EGF as described in Methods). Expression of hepatic (albumin) and biliary markers ($\cdot$GT, CK19 and aquaporin-1 [49]) was determined by qPCR and expressed as % relative to untreated control cells. After 5 days of differentiation, Gal-3(-/-) HPCs exhibited increased expression of both the biliary cell markers: CK19 (A), GGT (B), Aquaporin-1 (C) and the hepatocyte marker: albumin (D), indicating that HPCs require Gal-3 to maintain an undifferentiated phenotype on laminin. Further hepatocyte functional analysis showed significantly higher albumin secretion (E) and CYP1A2 activity (F) in Gal-3 siRNA transfected BMOLs. (n=3, *P<0.05;**P,0.01;***P<0.001).

Figure 7 – Macrophage derived Gal-3 stimulates HPC proliferation *in vitro* and *in vivo*. (A) Gal-3 was measured by ELISA in 24h culture supernatants from BMDMs and WT HPCs. (B) Control and Gal-3 siRNA transfected HPCs co-cultured with WT or Gal-3(-/-) BMDMs was measured by MTT assay. Inhibition of Gal-3 expression in BMOLs resulted in reduced proliferation which was partially restored when cells were cultured with WT but not Gal-3(-/-) BMDMs (n=3, *P<0.05).